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Development of a seaweed derived platelet activating factor acetylhydrolase (PAF-AH) inhibitory hydrolysate, synthesis of inhibitory peptides and assessment of their toxicity using the Zebrafish larvae assay

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ABSTRACT

The vascular inflammatory role of platelet activating factor acetylhydrolase (PAF-AH) is thought to be due to the formation of lysophosphatidyl choline and oxidized non-esterified fatty acids. This enzyme is considered a promising therapeutic target for the prevention of atherosclerosis and there is a need to expand the available chemical templates of PAF-AH inhibitors. This study demonstrated how natural PAF-AH inhibitory peptides were isolated and characterized from the red macroalga *Palmaria palmata*. The dried powdered alga was hydrolyzed using the food grade enzyme papain, and the resultant peptide containing fraction generated using RP-HPLC. Several oligopeptides were identified as potential PAF-AH inhibitors following bio-guided fractionation, and the amino acid sequences of these oligopeptides were confirmed by Q-TOF-MS and microwave-assisted solid phase *de novo* synthesis. The most promising PAF-AH inhibitory peptide had the amino acid sequence NIGK and a PAF-AH IC_{50} value of 2.32 mM. This peptide may constitute a valid drug template for PAF-AH inhibitors. Furthermore the *P. palmata* hydrolysate was nontoxic when assayed using the Zebrafish toxicity model at a concentration of 1 mg/ml.

1. Introduction

The red seaweed *Palmaria palmata* has a recorded protein content of up to 25% depending on seasonal and geographical variation [7]. This protein source may be used as a starting material for the generation of bioactive peptides using food grade enzymes. Bioactive peptides derived from plant, animal and marine proteins through hydrolysis using proteolytic enzymes were identified previously with the ability to inhibit enzymes such as angiotensin converting enzyme (ACE-I) [24] and renin [6,11]. However, to date, inhibition of platelet activating factor acetylhydrolase (PAF-AH) by bioactive peptides has not been reported.

Atherosclerosis is an inflammatory disease and the most common cause of stroke and cardiovascular disease worldwide [18]. Recent studies suggest that the enzyme lipoprotein-associated phospholipase A_2 (Lp-PLA_2) or platelet activating factor acetylhydrolase (PAF-AH) plays an active role in atherosclerotic development and progression [12,13,25]. Indeed, in 2008, Wilensky and colleagues showed that selective inhibition of PAF-AH prevented progression to advanced coronary atherosclerotic lesions and confirmed the role of vascular inflammation, independent from hypercholesterolemia, in the development of lesions implicated in the pathogenesis of myocardial infarction and stroke [25].

PAF-AH is secreted from multiple inflammatory cells but is highly expressed in the necrotic core of atherosclerotic lesions [3,21]. PAF-AH is predominately bound to apolipoprotein β-containing lipids and 80% of PAF-AH in blood plasma is bound to low density lipoproteins (LDL) [3,21]. LDL is oxidized following its transportation from the lumen to the intima of the cell [21]. This facilitates hydrolysis of LDL by the enzyme PAF-AH in to two pro-inflammatory mediators; lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acids (oxNEFAs) [17,25].

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Pharmaceutical companies such as GlaxoSmithKline (GSK) have invested financially in the development of synthetic compounds to combat atherosclerosis through PAF-AH inhibition. For example, GSK are in phase three clinical trials with the substituted pyrimidone Darapladib, a synthetic, non-peptidic PAF-AH inhibitor [5]. However very few natural inhibitors of PAF-AH are known to date. Yu et al. identified PAF-AH inhibitors from extracts generated from 224 terrestrial plan extracts. Methanolic extracts were generated from these plants and were further partitioned with *n*-hexane, chloroform and ethyl acetate [26]. Seven methanol extracts were found to inhibit PAF-AH by 50% at a concentration of 100 μg/ml and two ethyl acetate extracts were found to inhibit PAF-AH activity at the same concentration [26]. In another study, synthetic analogs of cyclic enol-carbamates isolated from the bacteria *Pseudomonas fluorescens* were also identified as potent inhibitors of PAF-AH [16].

In this study, the amino acid composition of Irish sourced *P. palmata* was investigated. Furthermore a PAF-AH inhibitory tetrapeptide with the amino acid sequence Asparagine-Isoleucine-Glycine-Lysine (NIGK) was generated, enriched and isolated from the red seaweed *P. palmata* by hydrolysis of this seaweed protein with the food grade enzyme papain. This enzyme was chosen as it was used successfully in previous studies to release bioactive peptides from protein. Papain is also a food grade cysteine protease with known proteolytic capabilities [6,27]. *P. palmata* was chosen as it is known to be rich in protein and was reported previously to contain up to 25% protein depending on the season of collection and the geographical location [7]. The peptide NIGK was chemically synthesized and its PAF-AH inhibitory activity confirmed using an *in vitro* bioassay method. In addition, the toxicity of the seaweed hydrolysate was assessed using the Zebrafish bioassay. Two-day-old Zebrafish larvae were exposed to 1, 5, and 10 mg/ml concentrations of the hydrolysate over a 48 h period to examine any toxic effects the hydrolysate may have on a living model.

2. Materials and methods

2.1. Materials and reagents

The PAF-AH inhibitor screening kit was supplied by the Cayman Chemical Company (Ann Arbor, MI, USA). Acetonitrile and water were supplied by Romil Ltd. (Cambridge, England, United Kingdom). Dimethyl sulfoxide (DMSO), ammonium sulphate and the specific platelet activating factor (PAF) inhibitor methyl arachidonyl fluorophosphates (MAFP), which was used as a positive control, were supplied by Sigma–Aldrich (Steinheim, Germany). H-Ala-HMPB-ChemMatrix and H-Ile-HMPB-ChemMatrix resins were supplied by PCAS Biomatrix Inc. (Quebec, Canada). All other chemicals used were of analytical grade.

2.2. P. palmata protein extraction

Crude protein was extracted from *P. palmata* following the protocol of Galland-Irmouli et al. [7]. The extraction process was carried out at 4 °C. To induce osmotic shock in plant cells, 10 g of dried and milled *P. palmata* was suspended in 1 L of ultrapure water. Following ultrasonication (Branson UltraSonic Bath Model 3510, Danbury, CT, USA) for an hour, the seaweed solution was left to stir overnight on a magnetic stirrer plate (C-MAGHS 7KAMAG®, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The solution was then centrifuged at 10,000 × *g* for 1 h in a Sigma 6K10 ultracentrifuge (Sigma–Aldrich Steinheim, Germany). The pellet was subjected to a secondary extraction by suspending it in 200 ml of ultrapure water and left to stir overnight at 4 °C. Both supernatants were pooled and brought to 80% ammonium sulphate saturation, stirred for an hour at 4 °C, and centrifuged at 20,000 × *g* for 1 h to precipitate the protein fraction. The precipitates were then dialyzed against water overnight using 3.5 kDa MWCO dialysis tubing (Fisher Scientific, New Hampshire State, USA). The precipitates were subsequently freeze-dried in an Edwards micromodulyo 4K freeze drier (Edwards, Sandborn, New York, USA) and stored at −80 °C.

2.3. Enzymatic hydrolysis of Seaweed protein

Papain hydrolysates (X 3) of the crude *P. palmata* protein were prepared using a New Brunswick 1.5 L bioreactor (Cambridge, UK) with temperature and pH control. A substrate solution was prepared by suspending the dried seaweed protein in Romil HPLC grade water at a concentration of 0.015 g/ml at a total volume of 1 L. The temperature was adjusted to 60 °C and the pH to 6.0. The pH was regulated using 0.01 M NaOH. Once the optimum conditions were achieved, the enzyme papain was added at a concentration of 20.7 U/mg protein to initiate hydrolysis. Temperature, stirring and pH were kept constant for 24 h. The hydrolysis was stopped by heating the mixture at 95 °C for 10 min in a water bath (Grant JB Aqua 12, Grant instruments, Cambridgeshire, UK).

2.4. PAF-AH inhibitor screening assay

This assay was carried out using a PAF-AH inhibitor kit supplied by Cayman Chemical Company in accordance with the manufacturers' instructions. Briefly, prior to carrying out the assay, the PAF-AH inhibitor assay buffer was diluted by adding 27 ml of HPLC grade water to 3 ml of 0.1 M Tris–HCl (pH 7.2). The substrate, 2-thio PAF was reconstituted in 12 ml of diluted assay buffer to achieve a concentration of 400 μM. Background wells were prepared by adding 10 μl of assay buffer, 200 μl of 2-thio PAF substrate and 10 μl of solvent (in this case DMSO/water (5:95, v/v)). Initial activity wells were prepared by adding 200 μl of the 2-thio PAF substrate solution and 10 μl of the solvent DMSO/water (5:95, v/v). Inhibitor wells were prepared by adding 200 μl of the 2-thio PAF substrate solution and 10 μl of the test fraction. The reaction was initiated by adding 10 μl of human plasma PAF-AH to all wells except the background wells. The plate was then covered and incubated for 20 min at 25 °C. After incubation, 10 μl of 5,5′ dithio bis (2 nitrobenzoic acid) DTNB was added to all wells to develop the reaction. All potential inhibitors were assessed in triplicate. MAFP, a known PAF-AH inhibitor was used as a positive control.

2.5. Enrichment of P. palmata hydrolysate using RP-HPLC

The *P. palmaria* papain hydrolysate was enriched further using the Varian Pro-Star Reverse phase high performance liquid chromatography (RP-HPLC) system coupled to a photodiode array detector (SpectraLab Scientific Inc., Ontario, Canada). Samples were first filtered using 0.22 μm filters (Millipore, Billerica, MA, USA) and injected at a concentration of 1 mg/ml on a reverse phase Phenomenex (Torrance, CA, USA) C18 column with 5 μm particle size (100 μm × 21.2 mm). This column was initially equilibrated with TFA/H_2O (0.1%, v/v) at a flow rate of 1.0 ml/min, before the concentration of the eluting solvent (TFA/acetonitrile (0.1%, v/v)) was raised from 0% to 100% over 60 min. Chromatogram peaks were integrated at an absorbance of 214 nm. Eluted fractions were collected every minute. Acetonitrile and TFA were removed under nitrogen and subsequently freeze dried.

2.6. Peptide identification by tandem mass spectrometry

The most active RP-HPLC fraction which was eluted after 25 min was further purified and analyzed using an electrospray ionization